

AD-A259 591

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4. TITLE AND SUBTITLE HIV Neutralization Assay Using Polymerase Chain Reaction-Derived Molecular Signals		5. FUNDING NUMBERS	
6. AUTHOR(S) Merlin L. Robb, Victoria Polonis, Maryanne Vahey, *Suzanne Gartner, Nelson Michael, °Arnold Fowler, and Robert Redfield			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Retroviral Research, Division of Retrovirology Walter Reed Army Institute of Research; *Henry M. Jackson Foundation; and °SRA Technologies, Rockville, Maryland, USA.		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research & Development Command Fort Detrick, Frederick, MD 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT APPROVED FOR PUBLIC RELEASE: DISTRIBUTION UNLIMITED		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  Summary: Characterization of the capacity of human polyclonal antibody to neutralize wild-type patient isolates has important implications for vaccine development. We report the development of a polymerase chain reaction-based neutralization assay that quantitatively measures each infection using HIV proviral formation. These molecular end points included the absence or quantitative diminution of DNA provirus formation as well as a delay in the kinetics of HIV DNA provirus formation. Using both laboratory strain prototype isolates (HIV-1-MN, HIV-1IIB) and primary wild-type patients' isolates, neutralization end points were reproducibly determined. End points were reached within 72 h, thereby minimizing the impact of subsequent rounds of infection on interpretation of results. Although the neutralization titer of polyclonal sera was usually comparable using standard technology, this assay did find isolate-dependent variation in the relationship between p24 production and HIV proviral DNA formation. Finally, we noted the disparity between the ability of human sera to neutralize prototype and wild-type isolates in primary peripheral blood mononuclear cell targets. We believe this assay provides unique opportunities to characterize the initial events of virus-antibody interaction and will help to elucidate clinically relevant neutralization immunoregulatory mechanisms. Key Words: HIV neutralization assay—Polymerase chain reaction—Polyclonal antibody—DNA provirus formation—Immunoregulatory mechanisms.			
14. SUBJECT TERMS HIV neutralization assay—Polymerase chain reaction—Polyclonal antibody—DNA provirus formation—Immunoregulatory mechanisms.		15. NUMBER OF PAGES	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

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NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## HIV Neutralization Assay Using Polymerase Chain Reaction-Derived Molecular Signals

Merlin L. Robb, Victoria Polonis, Maryanne Vahey, \*Suzanne Gartner,  
Nelson Michael, †Arnold Fowler, and Robert R. Redfield

Department of Retroviral Research, Division of Retrovirology, Walter Reed Army Institute of Research; \*Henry M. Jackson Foundation; and †SRA Technologies, Rockville, Maryland, U.S.A.

**Summary:** Characterization of the capacity of human polyclonal antibody to neutralize wild-type patient isolates has important implications for vaccine development. We report the development of a polymerase chain reaction-based neutralization assay that quantitatively measures each infection using HIV proviral formation. These molecular end points identified the absence or quantitative diminution of DNA provirus formation as well as a delay in the kinetics of HIV DNA provirus formation. Using both laboratory strain prototype isolates (HIV-1-MN, HIV-IIIb) and primary wild-type patients' isolates, neutralization end points were reproducibly determined. End points were reached within 72 h, thereby minimizing the impact of subsequent rounds of infection on interpretation of results. Although the neutralization titer of polyclonal sera was usually comparable using standard technology, this assay did find isolate-dependent variation in the relationship between p24 production and HIV proviral DNA formation. Finally, we noted the disparity between the ability of human sera to neutralize prototype and wild-type isolates in primary peripheral blood mononuclear cell targets. We believe this assay provides unique opportunities to characterize the initial events of virus-antibody interaction and will help to elucidate clinically relevant neutralization immunoregulatory mechanisms. **Key Words:** HIV neutralization assay—Polymerase chain reaction—Polyclonal antibody—DNA provirus formation—Immunoregulatory mechanisms.

The identification of serum antibodies that inhibit viral infection in vitro is a useful marker of protective immunity in vivo (1). However, the relationship between the appearance of neutralizing antibodies to human immunodeficiency virus (HIV-1) and a

beneficial clinical effect, with respect to either prevention of infection or protection from disease progression, is not well defined (2-8). A variety of experimental parameters may confound the relevance of an in vitro assay to in vivo events (9,10). Some of these parameters include viral strain selection, choice of assay target cell, method of detecting viral infection, and the time of end point determination (11,12).

Current methods for analyzing the effect of antibody on the replication of HIV-1 rely upon infection as defined by either the production of a viral-specific protein or the manifestation of a cytopathic effect. We have developed an assay using molecular end points derived with the polymerase chain reac-

Address correspondence and reprint requests to Dr. Merlin L. Robb at Department of Retroviral Research, Division of Retrovirology, Walter Reed Army Institute of Research, 13 Taft Ct., Rockville, MD 20850, U.S.A.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as necessarily reflecting the view of the Department of Defense of the Department of the Army.

Manuscript received January 26, 1992; accepted April 30, 1992.

tion to obtain a more accurate and relevant method of evaluating the impact of serum antibody on viral replication. The method reported here permits the use of HIV-1 derived directly from patients; the early and accurate recognition of viral infection, and the use of natural targets of infection. Infectious events were determined by quantifying the number of genomic DNA copies of HIV-1 arising in an *in vitro* population of stimulated peripheral blood mononuclear cells. The method was evaluated in a neutralization format using prototypic laboratory-adapted isolates, HIV-1-MN and HIV-1-IIIb, and two isolates derived from patients.

## MATERIALS AND METHODS

### Preparation of Viral Stocks and Cell Targets

Peripheral blood mononuclear cells (PBMCs) from a single HIV-1-negative donor were harvested and cryopreserved for use throughout the experiment. Cells were washed using complete RPMI (cRPMI), which was RPMI supplemented with 10% fetal calf serum, 50 U Pen/Strep (GIBCO) per ml, and 2 mM L-glutamine. The PBMC targets were thawed in batches of  $10 \times 10^6$  cells, washed, and suspended for 72 h in 5 ml of RPMI containing 20% fetal calf serum, 250 U Pen/Strep, 2 mM L-glutamine (GIBCO), and 5  $\mu$ g PHA-P (DIFCO). The cells were washed to remove PHA, pelleted at 250 g, and suspended in either viral stock inoculum or patient PBMC cell suspension (cocultivation) for 30 min at 37°C. After infection, the cells were washed and suspended at a density of  $1 \times 10^6$  cells per ml in T-cell growth media consisting of RPMI supplemented with PEN/STREP, L-glutamine, and 10% recombinant interleukin-2 (rIL-2) (Boehringer-Mannheim).

Patient isolates were recovered by cocultivation and propagated in PBMCs. Supernatants were filtered through a 0.22- $\mu$ m filter to ensure a cell-free stock. Lab-adapted isolates—HIV-1-IIIb and HIV-1-MN—were employed to provide a comparison with existing assay systems. These were prepared from infected H9 cells by 50-fold concentration of culture supernatant and filtration through a 0.22- $\mu$ m filter. All viral stocks were titered in PBMCs to determine the median tissue culture infectious dose (TCID<sub>50</sub>) based on production of p24 after 7 days in culture. In order to eliminate residual HIV-1 DNA contamination, the cell-free viral stocks were treated with RNase-free DNase (Boehringer-Mannheim) at a concentration of 50 U per ml of stock for 30 min at 21°C before use.

### Neutralization Assay

Viral stocks were mixed thoroughly with immune or nonimmune serum and incubated for 30 min on wet ice or at room temperature. HIV-1-negative donor PBMCs were incubated with the viral stock-serum dilution at 1, 20, 40, or 80  $\times$  TCID<sub>50</sub> for 30 min at 37°C. The cells were washed once with cRPMI and seeded into six wells of a 48-well microtiter plate at a concentration of 500,000 cells in 500  $\mu$ l of complete T-cell media per well. Sam-

ples were collected at the time of infection (time 0) and at 24, 48, and 72 h postinfection. One well was harvested for each time point. For comparison, neutralization was also performed with a syncytia inhibition assay using CEM-SS cell targets (13).

However, CEM-SS cannot support the growth of most patient isolates. Hence, neutralization of patient viral isolates in a polymerase chain reaction (PCR)-based assay was compared with a similar assay using p24 antigen end points. Briefly, this method uses patient viral isolates propagated in PBMCs, passed through a 0.22- $\mu$ m filter, and titered with PBMCs using p24 as the culture end point. Virus was mixed and incubated with an equal volume of sera for 30 min on wet ice or at room temperature. The virus/sera mixture was then incubated with target PBMCs to yield 40  $\times$  TCID<sub>50</sub> for 30 min at 37°C and washed to remove all p24. One million infected cells in 1 ml of complete T-cell media were seeded into a 24-well microtiter plate. Culture supernatants were harvested on days 3, 5, and 7 and analyzed by p24 antigen capture (Coulter).

### DNA Preparation and PCR

The cells were counted at each time point and pelleted at 250 g for 10 min, and the supernatant was removed. The cell pellet was suspended in 50  $\mu$ l of cRPMI and boiled for 15 min to destroy residual DNase. The crude lysate was treated with proteinase K (BRL) for 60 min at 56°C in 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub>, 0.45% Triton-X 100, and 0.45% Tween 20. Proteinase K was inactivated by boiling for 15 min. Lysates were analyzed by a quantitative PCR assay for HIV-1 DNA using primers from highly conserved sequences in the *gag* region. This region is the last segment of the RNA template to undergo reverse transcription and represents full-length genomic DNA (14). The sequence of the primer pairs and probes are defined by their position in HIV-1-MN according to the Los Alamos HIV-1 Sequence Databank: Primer set "00" and probe GPR-5: 5' primer, (1412-1438) CAATGAGGAAGCTGCAGAAATGGGATAG; 3' primer, (1523-1547) CCTTCAGGAACAAATAGGATGGATG; probe: (1479-1506) ATGAGAGAACCAAGGGGAAGTGACATAGC A; primer set "NN" and probe GPR-1: 5' primer, (1223-1250) GGTACATCAGGCCATATCACCTAGAAGT; 3' primer, (1412-1438) CTATCCCATTCTGCAGCTTCCTCATTG; probe, (1302-1337) ATACCCATGTTTTCAGCATTATCAGAAGGAGCCACC.

Each lysate was amplified in duplicate using a standard amplification buffer consisting of 200  $\mu$ M of each deoxynucleotide triphosphate, 1.64 mM Mg<sup>2+</sup>, 1  $\mu$ M of each primer, 2.5 U of *Taq* polymerase (Perkin-Elmer) per reaction tube, and 10  $\mu$ l of lysate containing ~0.5  $\mu$ g of cellular DNA. Each primer set was evaluated at a series of annealing temperatures and cycle routines to determine an optimal quantitative performance, as described below. Melting was always performed at 95°C for 30 s, and extension was performed at 72°C for 3 min without temperature ramping. The PCR products were separated through a 1.4% agarose gel and pressure-blotted to a nylon filter. The blot was hybridized with a <sup>32</sup>P end-labeled oligonucleotide probe. Signals were quantified with storage phosphor technology (Molecular Dynamics). The positive control for the assay was a plasmid construct that contained a portion of the *gag* sequence (14). A series of controls were coamplified with each experimental panel, providing a linear standard from five to 5,000 copies. The copy number present in the cell lysate was determined by interpolation of the

amplification product signal to the standard curve produced from the coamplified, cohybridized controls. The final HIV-1 DNA copy number was adjusted based on cell count at the time of lysate preparation to a standard of 10,000 PBMC targets. Verification of adequate amplifiable, cellular DNA was obtained by comparison of the  $\beta$ -globin DNA content of each sample, as determined by PCR with primers specific for this gene.

## RESULTS

The PCR conditions were optimized for each primer set by constructing annealing curves using the standard PCR buffer and a series of plasmid controls. Both primers reported here provided maximal target amplification at an annealing temperature of 55°C. The ability to quantify PCR reaction products accurately was most sensitive to amplification cycle number. Several cycle routines were evaluated, and a plateau of detectable product with a template of  $\geq 500$  copies per lane at 28 cycles of amplification was identified. Conversely, there was no detectable product at a five or 10 copy input with  $\leq 24$  cycles. Standard curves on all experiments were linear in the required range of input template with regression coefficients of  $\geq 0.94$ . Subsequently, all unknown samples were quantified using 26 cycles of amplification and an annealing temperature of 55°C.

A range of tissue culture infectious doses was examined with HIV-1-IIIB and HIV-1-MN to determine the time course of HIV-1 DNA formation. PCR product could be derived from all doses after 24 h of culture. However, the copy number obtained at  $1 \times \text{TCID}_{50}$  was relatively low throughout the time course of infection. In addition,  $1 \times \text{TCID}_{50}$  did not provide uniform product in replicate experiments, suggesting that insufficient infected cells were present to insure consistent distribution of infected cells for each time point. The  $80 \times \text{TCID}_{50}$ , on average, tended to exceed the linear range of our assay and proved difficult to achieve in patient isolates. Thus,  $40 \times \text{TCID}_{50}$  was chosen for subsequent experiments with neutralizing sera.

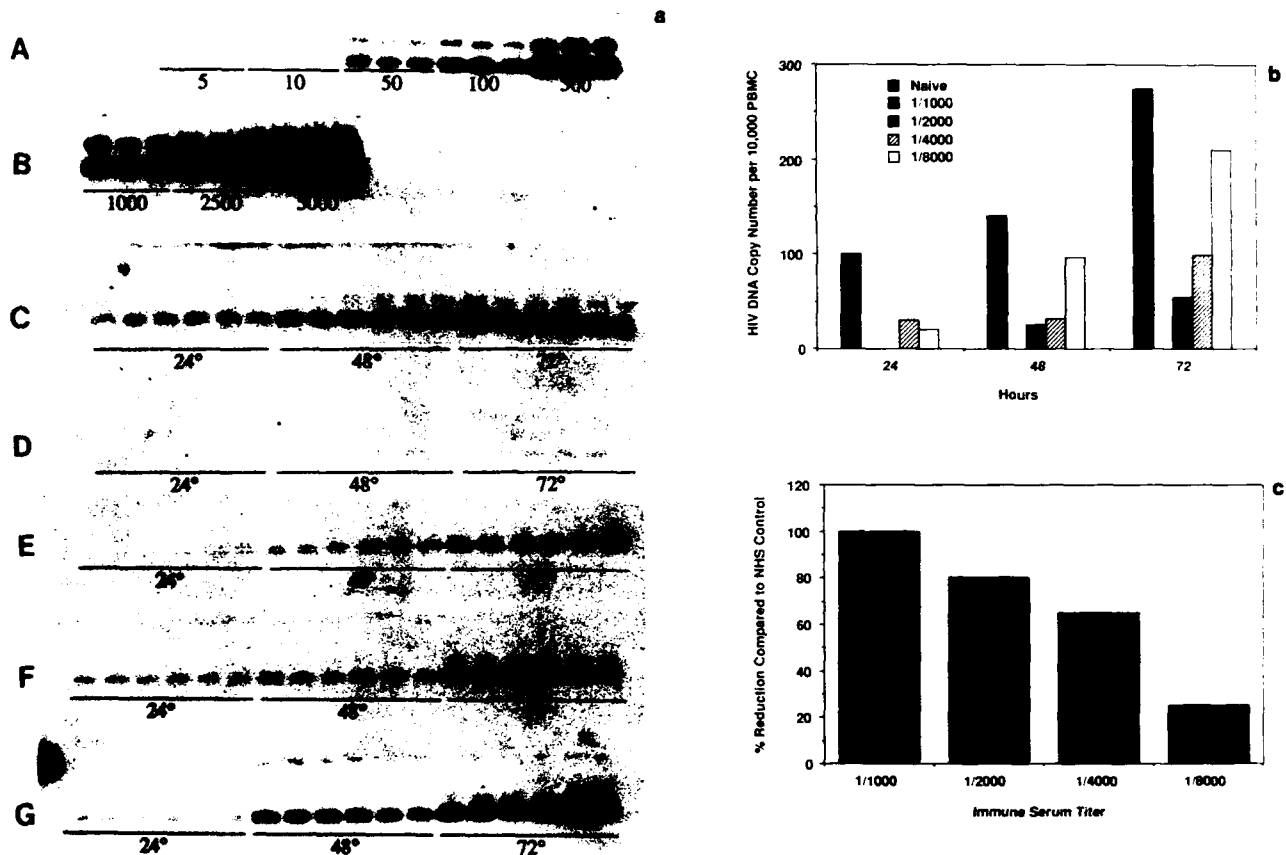
A well-characterized patient serum was employed to determine the impact of neutralization on observed HIV-1 DNA formation (Fig. 1a). HIV-1 DNA was found at 24 h in the normal human serum control (NHS) and increased from 100 copies per 10,000 PBMCs to 274 copies per 10,000 PBMCs after 72 h in culture (Fig. 1b). Comparison of HIV-1 DNA copy numbers in each of the immune serum dilutions to the normal human serum control yielded the percentage neutralization. A 90% reduc-

tion of HIV-1 infection was consistently noted for HIV-1-MN with reciprocal serum titers of  $\leq 1,000$ . Serum dilutions  $> 1,000$  exhibited earlier and quantitatively greater HIV-1 DNA signals. The percentage neutralization for reciprocal titers of 2,000, 4,000, and 8,000 were 80%, 65%, and 23%, respectively (Fig. 1c). There was no evidence of enhancement at any dilution tested.

Culture supernatants recovered at the time of cell harvest contained p24 antigen in the range of 13–65 pg/ml, which corresponded to the HIV-1 DNA signals detected by PCR. Although p24 antigen was detectable, it was less sensitive over the 72 h of culture than the PCR-derived HIV-1 DNA signals, since p24 antigen was entirely absent from all cultures with  $< 100$  copies of HIV-1 DNA per 10,000 PBMCs. The average production of p24 antigen was 15.7 fg per copy of HIV-1-MN DNA. The apparent neutralization titer at 72 h using a p24 antigen end point was 6,500, but at 7 days the titer was 517, possibly reflecting the growth of virions that escaped neutralization (Table 1). Alternatively, these findings may be due to delayed reverse transcription in a population of cells infected by the initial viral inoculum.

Two clinical isolates derived from patients with Walter Reed stage 1 HIV-1 infection (15), were propagated in PBMCs and titered after filtration of stocks. In contrast to the data from neutralization of MN, the patient isolates could not be neutralized at the  $\geq 90\%$  level despite the use of low immune serum dilutions. A comparison of 50% neutralization titers showed a reciprocal dilution of 100, 338, and 2,511 for patient 1, patient 7, and MN virus, respectively (Table 1). Culture supernatants from patient 1 were assayed for p24 antigen, and levels of 108–687 pg/ml were found. In contrast to the experiment with MN, there was detectable p24 in cultures with as few as eight copies of HIV-1 DNA per 10,000 PBMCs, and p24 production was, on average, 140 fg per copy of HIV-1 DNA. Although there was an approximately ninefold variation in p24 antigen production per HIV-1 copy, there was greater similarity of HIV-1 copy number between isolates  $40 \times \text{TCID}_{50}$ . After 72 h of culture there were 274 and 350 copies per 10,000 PBMCs for MN and patient 1 virus, respectively.

A comparison of three neutralization methods for the three neutralization experiments reported here shows a decline in 50% neutralization titer for experiments that acquire end points after longer periods of culture (Table 1). A comparison of the p24



**FIG. 1.** Neutralization of HIV-1-MN by patient sera. **a:** Phosphor image of Southern blot of plasmid controls from five to 5,000 copies per lane and three replicates per control (rows A and B), normal human serum control at a reciprocal dilution of 200 with six replicates per time point (row C), and immune serum at reciprocal dilutions of 1,000 (row D), 2,000 (row E), 4,000 (row F), and 8,000 (row G). **b:** Mean HIV-1 DNA copy number for the normal human serum control and each dilution of immune serum at 24-, 48-, and 72-h time collections. **c:** Percentage neutralization for each immune serum dilution determined by reduction of DNA copy number in immune serum compared with the normal human control at 72 h.

antigen end points with PCR-derived end points shows that in almost every case the observed 50% neutralization titer was higher using molecular end points and shorter culture periods. The single exception was the high neutralization titer with MN in PBMCs at 3 days of culture due to the very low p24 signal obtained for samples with significant HIV-1 DNA formation.

## DISCUSSION

The evaluation of the effect of serum antibody on the neutralization of virus has been a powerful tool in vaccine development. Historically, these assays have employed laboratory-adapted strains of virus and cell lines that provide a basis for identifying antibody responses that confer protection from dis-

ease in vivo (1). The role of neutralizing antibodies in the pathogenesis and prevention of HIV-1 infection, however, has been controversial (2-8,14). Unlike other viral diseases, there is a well-recognized genomic and biological heterogeneity of HIV-1 within an infected individual, which develops quickly after infection and continues throughout the course of infection (15-19). In order to assess the role antibody plays in modulating HIV-1 disease progression, it may be necessary to determine the influence of serum antibodies on the population of viruses present in the patient at a given time. Moreover, vaccine development will require methods to identify quantitatively the activity of a particular antibody or serum against a variety of viral isolates to determine if type-specific or group-specific protection is possible.

**TABLE 1.** Comparison of PCR-based neutralization to an autologous assay using p24 antigen capture and CEM-SS syncytia inhibition assay

Assays	Virus MN vs. serum IM	Virus and sera (pt. 1)	Virus and sera (pt. 7)
PCR-based neutralization	1/2,500 1/1,000 <sup>a</sup>	1/100	1/338
Autologous neutralization using p24			
Day 3	1/6,500	1/61	1/160-1/320
Day 7	1/517	1/25	<1/40
CEM-SS-based syncytia inhibition	1/1,024 <sup>a</sup>	Not done	Not done

MN an IM, HIV-MN- and IM-HIV-positive patient serum, respectively.

All titers represent 50% reduction compared with normal human serum.

<sup>a</sup>A 90% reduction was recorded.

An HIV-1 neutralization assay must therefore meet two requirements. The assay must impose a minimum selection pressure on the viral isolates, thus permitting a complete representation of *in vivo* diversity. Second, it must provide a quantitative end point that accurately reflects infectious events, thus allowing comparison of several sera against a reference virus as well as comparison of several isolates of HIV-1 against a reference serum. The data presented here indicate that a PCR-based assay of the early events in viral infection meets these requirements and can be quantitatively performed over a wide range of infectious doses. The use of molecular end points has the advantage of providing a rapid assay using natural targets of infection and isolates derived directly from patients.

Most of the neutralization data reported for HIV-1 has been derived from assays that rely on the use of cell lines, laboratory-adapted strains of HIV-1, or nonquantitative end points (16,20). Cell line-adapted strains of HIV-1 may not be representative of prevalent field isolates by virtue of both their genotypic divergence as well as their biological properties of syncytia formation and cell line adaptation. The use of transformed cell lines limits the use of some patient isolates and may impose a selection bias. The extent to which this bias occurs has been characterized by sequence analysis of the viral genome and description of the phenotype (11,12). Assays that rely on HIV-1 protein production or cell viability do not enumerate the number of infections in a cell culture (16). Such assays cannot permit a comparison of an antibody or serum against a collection of viral isolates unless the stan-

dard of neutralization is 100%. Assay systems that rely upon immunofluorescent antibody staining of infected cells are quantitative but are difficult to interpret on such natural targets as PBMCs, since so few of the cells are infected. Classic plaque reduction assays have been difficult to establish in HIV-1. Moreover, it is possible that formation of syncytia or plaques may not accurately reflect the number of infectious events, thus rendering comparisons of one serum against several viruses problematic.

An autologous assay that employs patient isolates and PBMC targets has been developed (4,5). This method uses p24 antigen production as the marker of infection. However, the data presented in this study showed significant variation between a patient isolate and the HIV-1-MN isolate with respect to the amount of p24 antigen production for each copy of viral DNA despite biological equivalence, i.e.,  $40 \times \text{TCID}_{50}$ . This observation corresponds to previous data that showed that minimal infectious doses of different HIV-1 isolates had markedly different p24 antigen contents (21). The lack of a consistent stoichiometric relationship between p24 antigen production and infectivity limits the ability to compare a given serum against several different viral isolates using p24 antigen as the experimental end point.

The PCR-based neutralization assay is quantitative because it recognizes a unique infectious event through the identification of a new copy of HIV-1 DNA. The selection of *gag* DNA as the target of amplification ensures that reverse transcription has been virtually completed, thus approximating the number of proviral copies in a cell population. It is possible that some unknown proportion of reverse-transcribed HIV-1 DNA fails to result in productive infection, but this limitation is not relevant to a system designed to assess the impact of antibody on the initial events of infection.

Use of quantitative methods such as those described here are critical to an accurate understanding of the impact of immune sera on a complex mixture of HIV-1 strains present in a single patient isolate. The foregoing considerations are equally applicable to the assessment of other interventions, such as zidovudine (ZDV) or new antiretroviral agents. As methods of automating PCR and PCR product quantification become available, a system of measuring the number of infectious events becomes attractive for drug screening and susceptibility testing. A PCR-based neutralization assay may

permit a useful analysis of the role of antibody on the initial molecular events of viral infection and thus complement existing assays of neutralization.

**Acknowledgment:** We thank Donald Burke for his support and encouragement of this study and Nathan Caffo, Maria Wood, and Linda James for their technical assistance.

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